

Amendments to the Specification:

Please replace the paragraph on page 8, line 4, with the following rewritten paragraph:

--The present invention further provides a method for the expression of a heterologous polypeptide by a host cell in association with an oil body and separating said heterologous polypeptide from the oil body, said method comprising: a) transforming a first host cell with a first chimeric nucleic acid sequence comprising: 1) a first nucleic acid ~~ais~~ acid sequence capable of regulating the transcription in said host cell of 2) a second nucleic acid sequence, wherein said second sequence encodes a first fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oil body protein gene to provide targeting of the first fusion polypeptide to a lipid phase linked in reading frame to (ii) a nucleic acid sequence encoding the heterologous polypeptide; and (iii) a linker nucleic acid sequence encoding an amino acid sequence that is specifically cleavable by enzymatic means wherein said linker nucleic acid sequence (iii) is located between said (i) nucleic acid sequence encoding the oil body protein and said (ii) nucleic acid sequence encoding the heterologous polypeptide; and 3) a third nucleic acid sequence encoding a termination region functional in the host cell; and b) transforming a second host cell with a second chimeric nucleic acid sequence comprising: 1) a first nucleic acid sequence capable of regulating the transcription specifically during seed germination and seed growth of 2) a second nucleic acid sequence wherein said second sequence encodes a second fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oil body protein gene to provide targeting of the second fusion polypeptide to a lipid phase linked in reading frame to a nucleic acid sequence, encoding a specific enzyme that is capable of cleaving the linker nucleic acid sequence of said first chimeric nucleic acid sequence; and 3) a third nucleic acid sequence encoding a termination region; c) growing said first host cell under conditions such that the first fusion polypeptide is expressed and associated with the oil bodies to produce a first oil body fraction containing the first recombinant fusion polypeptide; d) growing said second host cell under conditions such that the second fusion polypeptide is expressed and associated with the oil bodies to product a second oil body fraction containing the second

recombinant fusion polypeptide; e) contacting the first oil body fraction of step (c) with the second oil body fraction of step (d) under conditions such that the enzyme portion of the second fusion polypeptide cleaves the heterologous polypeptide from the first fusion polypeptide.--

Please replace the paragraph on page 9, line 21, with the following rewritten paragraph:

--The invention further provides methods for the separation of heterologous proteins from host cell components by partitioning of the oil body fraction and subsequent release of the heterologous protein via specific cleavage of the heterologous protein - oil body protein fusion. Optionally a cleavage site may be located prior to the N-terminus and after the C-terminus of the heterologous polypeptide allowing the fusion polypeptide to be cleaved and separated by phase separation into its component peptides. This production system finds utility in the production of many proteins and peptides such as those with pharmaceutical, ~~enzymic~~enzymatic, rheological and adhesive properties.--

Please replace the paragraph on page 12, line 19, with the following rewritten paragraph:

--Figure 6 shows the nucleotide sequence (SEQ.ID.NO.6) and deduced amino acid (SEQ.ID.NO.7 and NO.8) sequence of the 2.7 kbp *HindIII* fragment of pSBSOTPTNT containing the oleosin-chymosin fusion gene. Indicated in bold (nt 1625-1631) is the *NcoI* site containing the methionine start codon of the prochymosin sequence. The preceding spacer sequence (nt 1608-1630), replacing the oleosin stop_ codon is underlined.--

Please replace the paragraph on page 13, line 8, with the following rewritten paragraph:

--Figure 10. Nucleotide sequence of insert of pSBS2098 containing the phaseolin promoter- β Glucuronidase (GUS)-phaseolin terminator sequence (SEQ ID NO.36). The GUS sequence and its deduced amino acid sequence (SEQ ID NO.37) is indicated in uppercase. The phaseolin promoter corresponds to nucleotide 1-1547, and the

phaseolin terminator corresponds to nucleotide sequence 3426-4646. The terminator was furnished with a a KpnI site (nt 4647-4652) to facilitate cloning.--

Please replace the paragraph on page 13, line 15, with the following rewritten paragraph:

--Figure 11. Nucleotide sequence of the phaseolin promoter-oleosinGUS-phaseolin terminator sequence (SEQ ID NO.38). The oleosinGUS coding sequence and its deduced amino_acid sequence is indicated (SEQ ID NOs.39 and 40). The phaseolin promoter corresponds to nucleotide 6-1554. The sequence encoding oleosin corresponds to nt 1555-2313, the intron in this sequence (nt 1908-2147) is indicated in italics. The GUS sequence corresponds to nt 2314-4191. The phaseolin terminator corresponds to nucleotide sequence 4192-5412.--

Please replace the paragraph on page 13, line 23, with the following rewritten paragraph:

--Figure 12. Nucleotide sequence of the phaseolin promoter-caleosinGUS-phaseolin terminator sequence (SEQ ID NO.41). The caleosinGUS coding sequence and its deduced amino_acid sequence is indicated (SEQ ID NO.42). The phaseolin promoter corresponds to nucleotide 1-1545. The sequence encoding caleosin corresponds to nt 1548-2282, the NcoI restriction, which was used for the in-frame cloning and which separates the caleosin and GUS sequence (nt 2284-2289) is underlined. The GUS sequence corresponds to nt 2286-4163. The phaseolin terminator corresponds to nucleotide sequence 4164-5384.--

Please replace the paragraph on page 22, line 8, with the following rewritten paragraph:

--The degree of evolutionary relationship between the plant species chosen for isolation of a promoter and the plant species selected to carry out the invention may not be critical. The universality of most plant genes and promoter function within dicotyledonous species has been amply demonstrated in the literature. Additionally to a certain extent the conservation of function between monocot and dicot genes has also been shown. This is apparent to a person skilled in the art that the function of any given

promoter in any chosen species may be tested prior to ~~practising~~practicing the invention by simple means such as transient expression of marker gene promoter fusions in isolated cells or intact tissues. The promoter region typically comprises minimally from 100 bp 5' to the translational start of the structural gene coding sequence, up to 2.5 kb 5' from the same translational start.--

Please replace the paragraph on page 22, line 20, with the following rewritten paragraph:

--Examples of nucleic acid encoding sequences capable of providing targeting to an oil body protein are oleosins genes obtainable from *Arabidopsis thaliana* or *Brassica napus* which provide for expression of the protein of interest in seed (See Taylor et al., 1990, Planta 181:18-26). The necessary regions and amino-acid sequences needed to provide targeting to the oil body reside in the highly hydrophobic central region of oil body proteins. The amino acid sequence necessary to provide targeting to the oil body for *Arabidopsis thaliana* oleosins contain amino acids 46-117 shown in SEQ.ID.NO.2. ~~Similarly~~Similarly, the amino acid sequence necessary to provide targeting to the oil body for *Brassica napus* oleosins contains amino acids 60-132 shown in SEQ.ID.NO.5. In a preferred embodiment, the amino acid sequence necessary for targeting additionally contains the N-terminus of the oleosin which includes amino acids 1-45 (SEQ.ID.NO.2) and 1-60 (SEQ.ID.NO.5) for *Arabidopsis* and *Brassica*, respectively.--

Please replace the paragraph on page 26, line 7, with the following rewritten paragraph:

--Ligation of the nucleic acid sequence encoding the targeting sequence to the gene encoding the polypeptide of interest may take place in various ways including terminal fusions, internal fusions, and polymeric fusions. In all cases, the fusions are made to avoid disruption of the correct reading frame of the oil-body protein and to avoid inclusion of any translational stop signals in or near the junctions. The different types of terminal ~~an~~and internal fusions are shown in Figure_1 along with a representation of configurations *in vivo*.--

Please replace the paragraph on page 27, line 26, with the following rewritten paragraph:

--The mode by which the oil body protein and the protein to be expressed are fused can be either a N-terminal, C-terminal or internal fusion. The choice is dependant upon the application. For example, C-terminal fusions can be made as follows: A genomic clone of an oil body protein gene preferably containing at least 100 bp 5'to the translational start is cloned into a plasmid vehicle capable of replication in a suitable bacterial host (e.g., pUC or pBR322 in *E. coli*). A restriction site is located in the region encoding the hydrophilic C-terminal portion of gene. In a plant oil body protein of approximately 18 KDa, such as the *Arabidopsis* oleosin, this region stretches typically from codons 125 to the end of the clone. The ideal restriction site is unique, but this is not absolutely essential. If no convenient restriction site is located in this region, one may be introduced by site-directed mutagenesis. The only major restriction on the introduction of this site is that it must be placed 5' to the translational stop signal of the OBP clone.--

Please replace the paragraph on page 38, line 18, with the following rewritten paragraph:

Example 1: Isolation of Plant Oleosin Gene. Oil body proteins can be isolated from a variety of sources. The isolation of an oil body protein gene termed oleosin from the plant species *Arabidopsis thaliana* is described herein. Similar methods may be used by a person skilled in the art to isolate oil body proteins from other sources. In this example, a *Brassica napus* oleosin gene (described by Murphy et al, 1991, Biochim Biophys Acta 1088:86-94) was used to screen a genomic library of *A. thaliana* (cv. Columbia) constructed in the Lamda cloning vector EMBL-3A (Obtained from Stratagene Laboratories) using standard techniques. The screening resulted in the isolation of a an EMBL 3A clone (referred to as clone I2.1) containing a 15 kb genomic fragment which contains a oleosin gene from *A. thaliana*. The oleosin gene coding region is contained within a 6.6 kb *Kpn I* restriction fragment of this 15 kb fragment. The 6.6kb *Kpn I* restriction fragment was further mapped and a 1.8 kb *Nco I / Kpn I* fragment containing the oleosin gene including approximately 850 nucleotides of 5' sequence, the

complete coding sequence and the 3' region was isolated. This 1.8 kb fragment was end filled and subcloned in the *Sma* I site of RFM13mp19. The 1.8 kb insert was further digested with a number of standard restriction enzymes and subcloned in M13mp19 for sequencing. Standard cloning procedures were carried out according to Sambrook et al. (Molecular Cloning: A Laboratory Manual 2nd ed., 1989, Cold Spring Harbour Laboratory Press.) The nucleotide sequence was determined and the 1.8 kb sequence of the *A. thaliana* oleosin gene is presented in Figure 2 and SEQ ID No.1. This particular DNA sequence codes for a 18 KDa *A. thaliana* oleosin gene. The coding region contains a single intron. This gene was used for the construction of recombinant protein expression vectors. The gene may also be used for screening of genomic libraries of other species.--

Please replace the paragraph on page 42, line 23, with the following rewritten paragraph:

--The promoter region contained within the plasmid pUC OLEOp800 was fused to the reporter gene GUS. This was accomplished by substituting the oleosin promoter region for a heat shock promoter fused to a GUS gene in the plasmid HspGUS1559. HspGUS1559 is a plasmid used as a binary vector in *Agrobacterium*, derived from the vector pCGN 1559 (MacBride and Summerfeldt, 1990, Plant Molecular Biology, 14, 269-276) with an insert containing heat shock promoter (flanked by *Bam* *H1* sites), the β -glucuronidase open reading frame and a nopaline synthase terminator (derived from pB1221, Jefferson RA in Cloning Vectors 1988, Eds. Pouwels P., Enger-Valk BE, Brammer WJ., Elsevier Science Pub BV, Amsterdam section VII, Ai11). The binary plasmid HspGUS1559 was digested with *Bam**H1* which resulted in the release of the heat shock promoter and permitted the insertion of a *Bam**H1* fragment in its place. pUC OLEOp800 was then cut with *Bam* *H1* to yield a promoter fragment flanked by *Bam* *H1* sites. This fragment was cloned into the *Bam* *H1* sites of the plasmid HspGUS1559 to yield the *Agrobacterium* binary transformation vector pOLEOp800GUS1559. The other constructs were prepared by the same PCR method described above using the appropriate primers for amplifying the 2500 fragment, the -1200 fragment, the -600 fragment or the -200 fragment. These plasmids ~~was~~were used to transform *Brassica*

napus and tobacco. GUS expression assays (Jefferson R.A., 1987, Plant Mol. Biol. Rep. 5 387-405) were performed on the developing seeds and on non-reproductive plant parts as controls. The results in *Brassica napus* expressed as specific activity of GUS enzyme are shown in Table I. The results in tobacco are shown in Table II. GUS expression reported is an average obtained from approximately five seeds from each of approximately five different transgenic plants.--

Please replace the paragraph on page 52, line 28, with the following rewritten paragraph:

---This second oligonucleotide also encodes four amino acids specifying the amino acid sequence I-E-G-R, the recognition site for the endoprotease activity of factor Xa. The ~~ampli-fication~~ amplification product of approximately 1.8 kb comprises a GUS coding region flanked by a *Cla* 1 site at the 5' end and in place of the GUS termination codon, a short nucleotide sequence encoding the four amino acids that comprise the Factor Xa endoprotease activity cleavage site. Following these amino acid codons is a restriction site for *Bam*H1.--

Please replace the paragraph on page 60, line 13, with the following rewritten paragraph:

--Seeds from transgenic *B. napus* plants were ~~analysed~~ analyzed for cGH expression by Western blotting using monoclonal antibodies against cGH. The expected 40 kDa oleosin-cGH fusion protein was specifically detected in oil body protein extracts containing the oleosin-cGH fusion protein. A 22 kDa polypeptide corresponding with cGH could be released from oil bodies upon treatment with thrombin, while no cGH was detected in oil body protein extracts from untransformed control plants.--

Please replace the paragraph on page 65, line 4, with the following rewritten paragraph:

--Western blotting using a polyclonal anti-oleosin antibody revealed the presence of a 90 kDa polypeptide, which is in agreement with the molecular weight deduced from the amino acid sequence of the fusion protein (89.7 kDa). No cross-reactivity was observed in extracts from the untransformed strain or in extract transformed with the

control plasmid pM1830. Significant GUS activity could be detected in *S. cerevisiae* cells transformed with pM1830OleoGUS, while no ~~appriciable~~appreciable levels of GUS activity were measured in untransformed cells or cells transformed with pM1830 (table VIII).--

Please replace the paragraph on page 70, line 14, with the following rewritten paragraph:

--c) **Expression of a Oleosin/Phytase fusion protein in *B. napus*.** A microbial phytase from ~~a~~an *Aspergillus* may be isolated based on the published sequence (van Gorcom et al, European Patent Application 90202565.9, publication number 0 420 358 A1). This gene can be fused to the carboxy terminus of the oleosin protein using techniques described above and a collagenase recognition protease cleave site may be included to allow for separation of the phytase from the oil body if desired. The construct may contain, in the following order, the promoter region of the *Arabidopsis* oleosin gene, the coding sequence of the oleosin protein including the intron, a collagenase cleavage site and the phytase gene followed by the nos terminator polyadenylation signal. The construct can be inserted into the binary plasmid Bin 19 and the resultant plasmid introduced into *Agrobacterium*. The resulting strain can be used to transform *B.napus*. The seed of the transgenic plants will contain phytase activity. The phytase activity will be associated with the oil body fraction. The phytase activity is useful for the enhancement of meal for monogastric animal feed. The phytase may be purified by treatment with collagenase as described in a), or the transgenic seed may be used as a feed additive.--

Please replace the paragraph on page 71, line 4, with the following rewritten paragraph:

--d) **Expression of a an Oleosin/Glucose isomerase.** The enzyme glucose isomerase can be expressed as a oleosin fusion protein by joining the coding sequence for the enzyme, (for example, described by Wilhelm Hollenberg, 1985, Nucl. Acid. Res. 13:5717-5722) to the oleosin protein as described above. The construct may be used to transform *B. napus*.--

Please replace the paragraph on page 71, line 9, with the following rewritten paragraph:

--e) Expression of a an Oleosin/High Lysine Fusion Protein. In order to increase the lysine content of transgenic seeds, a polylysine oligonucleotide may be added to the 3' coding region of the oleosin gene. For example, a repetitive oligonucleotide encoding a polylysine coding sequence can be made by synthesizing a (AAG)₂₀ oligonucleotide that is joined to the 3' coding region of the oleosin gene by replacement of the hirudin coding sequence contained within pCBOGHIRT plasmid described above in example-8 with the polylysine oligonucleotide through the use of cohesive restriction termini. The construct may contain, in the following order, the promoter region of the *Arabidopsis* oleosin gene, the coding sequence of the oleosin protein including the intron, 20 codons for the amino acid lysine followed by the nos terminator polyadenylation signal. The construct may be inserted into the binary plasmid Bin 19 and the resultant plasmid may be introduced into the *Agrobacterium*. The resulting strain can be used to transform *B. napus*.--

Please replace the paragraph on page 71, line 23, with the following rewritten paragraph:

--f) Expression of an a Fungicidal Protein as an Oleosin Fusion Protein. As a further example of the invention, a oleosin fusion protein may be constructed which encodes a protein that is toxic to fungi. For example, the gene for the enzyme chitinase isolated from tobacco (Melchers et al, 1994, Plant Journal 5:469-480) may be fused to the 3' coding region of oleosin under the control of the native oleosin promoter. Included in this construct may be an oligonucleotide that encodes a collagenase recognition site located between the oleosin and chitinase coding regions. The expression of this construct will result in the production of a oleosin/chitinase fusion protein from which the chitinase enzyme can be released from the oleosin by treatment with collagenase. To this construct may be added a second chimeric gene capable of expression of a collagenase enzyme during seed germination. This second gene can comprise approximately 1.5 Kb of the 5' promoter region for isocitrate lyase, the collagenase coding sequence of *Vibrio alginolyticus* (Takeuchi et al., 1992, Biochemical Journal, 281:703-708) and the nos terminator. Isocitrate lyase is a glyoxysomal enzyme

expressed under transcriptional control during early stages of seed germination (Comai et al., 1989, *The Plant Cell*, 1:293-300). This second construct therefore will express collagenase during the germination of the seed and mobilization of the oil body reserves. Expression of isocitrate lyase is restricted to germination and is not expressed in developing seeds. This second gene, joined to the oleosin/chitinase gene can be inserted into the binary vector Bin 19. The resultant vector may be introduced into *Agrobacterium* and used to transform *Brassica napus* plants. It is noted that the two genes may also be introduced independently or in two different plants which are then combined through sexual crossing. Seed from transgenic plants would be collected and tested for resistance to fungi.--

Please replace the paragraph on page 72, line 21, with the following rewritten paragraph:

--g) **Expression of an Oleosin Fusion Protein that Provides Protection from Insect Predation.** As a further example of the invention, a fusion oleosin protein may be constructed which encodes a protein toxic to foraging insects. For example, the gene for cowpea trypsin inhibitor (Hilder et al., 1987, *Nature*, 330:160-163) may be used to replace the chitinase gene described in e). The expression of this construct will result in the production of a an oleosin/trypsin inhibitor fusion protein from which the trypsin inhibitor can be released from the oleosin by treatment with collagenase. By replacement of the chitinase gene in e) with the trypsin inhibitor, the construct also contains the collagenase gene under control of the germination specific promoter from the isocitrate lyase gene. This construct may be inserted into the binary vector Bin-19. The resultant vector can be introduced into *Agrobacterium* and used to transform *Brassica napus* plants. Seed from transgenic plants were collected and tested for resistance to insect predation.--

Please replace the paragraph on page 73, line 27, with the following rewritten paragraph:

--Expression of an oleosin/GUS fusion in mammalian cells would require the cloning of the GUS gene as described in example 17 in commercially available

mammalian expression vectors. For example, mammalian expression vectors pMSG, pSVL SV40, pCH 110, (all available from Pharmacia code No. 27-4506-01, 27-4509-01 and 27-4508-1 respectively) may be used. The oleosin/GUS fusion gene may be fused in the plasmid. These plasmids can be introduced into mammalian cells using established protocols (See eg. Introduction of DNA into mammalian cells (1995) Current Protocols in Molecular Biology, Ausubel et al. (ed) Supplement 29, Section 9). Accumulation of the oleosin/GUS transcript in mammalian cells can be determined after preparation of mammalian cell RNA (See eg. Direct analysis of RNA after transfection (1995) Current Protocols in Molecular Biology, Ausubel et al. (ed) Supplement 29, Section 9.8), northern blotting, and hybridization of this northern blot to a ³²P labelled labeled *Brassica* oleosin cDNA as described in Example 18. After preparation of a total protein extract from the transfected mammalian cell culture, GUS activity can be measured, demonstrating the accumulation of the oleosin/GUS protein. Alternatively, immunoblotting can be performed on this protein extract using commercially available GUS antibodies and/or oleosin antibodies.--